

Scaffold proteins LACK and TRACK as potential drug targets in kinetoplastid parasites: Development of inhibitors



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ABSTRACT

Parasitic diseases cause ~500,000 deaths annually and remain a major challenge for therapeutic development. Using a rational design based approach, we developed peptide inhibitors with anti-parasitic activity that were derived from the sequences of parasite scaffold proteins LACK (*Leishmania's* receptor for activated C-kinase) and TRACK (*Trypanosoma* receptor for activated C-kinase). We hypothesized that sequences in LACK and TRACK that are conserved in the parasites, but not in the mammalian ortholog, RACK (Receptor for activated C-kinase), may be interaction sites for signaling proteins that are critical for the parasites' viability. One of these peptides exhibited leishmanicidal and trypanocidal activity in culture. Moreover, in infected mice, this peptide was also effective in reducing parasitemia and increasing survival without toxic effects. The identified peptide is a promising new anti-parasitic drug lead, as its unique features may limit toxicity and drug-resistance, thus overcoming central limitations of most anti-parasitic drugs.

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1. Introduction

Leishmaniasis is one of the most neglected tropical diseases in terms of drug discovery and development. In over 88 countries, *Leishmania* threatens millions of the poorest people, especially those living in rural areas. Up to two million people are infected with *Leishmania* each year, and 70% of those who die from leishmaniasis are children (Chappuis et al., 2007). *Leishmania donovani*

(*L. donovani*) causes visceral leishmaniasis (VL), the most severe form of the disease in humans (Hotez et al., 2007). *Leishmania amazonensis* (*L. amazonensis*) causes cutaneous leishmaniasis (CL), the most common form of leishmaniasis in the New World (Guimaraes-Costa et al., 2009). CL is endemic in 82 countries, with an incidence of approximately 1.5 million cases per year.

Available treatments for VL and CL include Amphotericin B (a toxin that forms pores in membranes with some preferences for ergosterol (Croft et al., 2006)), with some success on the Indian subcontinent (Chappuis et al., 2007). In contrast to India, the use of Amphotericin B for VL in East Africa generally results in less-effective responses; thus, in this region, pentavalent antimonials, such as sodium stibogluconate (mode of action is not clearly

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understood (Copeland and Aronson, 2015)), are the first line of treatment. However, these drugs are highly toxic and cause serious side effects including hypotension, seizures, disturbances in cardiac conduction, and phlebototoxicity (vein inflammation, which limits repeated dosing (Croft and Coombs, 2003)). Moreover, widespread resistance has developed to these drugs. In March 2014, the FDA approved miltefosine (developed in the late 1980s for cancer treatment (Vincent et al., 2014)) for treatment of CL and VL caused by specific *Leishmania* species. Although initially miltefosine demonstrated high efficacy for VL in India, numerous clinical failures have since been reported. In addition, only moderate efficacy has been observed in East Africa and the New World (Monge-Maillo and López-Vélez, 2015).

Trypanosoma cruzi (*T. cruzi*) infection causes Chagas disease, affecting ~10 million people in the Americas, and is responsible for 12,000 deaths yearly (Bonney, 2014). Moreover, increased migration has disseminated this disease worldwide (Bonney, 2014), and about 70 million people, the majority of which are children, remain at high risk for contracting this disease (Hotez et al., 2012). During the acute phase after *T. cruzi* infection, clinical signs are usually quite minimal; however, many years after the primary infection, about 30–40% of infected individuals develop the symptomatic chronic phase of Chagas disease, characterized by the presence of myocarditis and heart failure (García et al., 2005). The drugs used to treat Chagas disease, benznidazole (which causes double-stranded breaks in DNA (Viotti et al., 2009)) and nifurtimox (mechanism of action is not yet fully elucidated), have limited use, as they have severe side effects and exhibit inadequate efficacy in the chronic stage (Maya et al., 2007). Several clinical studies found that although benznidazole treatment is somewhat beneficial, its side effects are still an issue (e.g. clinical study, NCT01162967, in which the treatment of 20% of the patients in the benznidazole group was discontinued because of severe cutaneous reactions (Molina et al., 2014)). Therefore, novel and simple strategies are needed for developing therapeutic agents that are effective and less toxic, do not trigger resistance, and are affordable for the infected populations in the developing world.

Here, we describe our effort to develop novel inhibitors for the parasites *Leishmania* sp. and *T. cruzi*. We concentrated on generating inhibitors that are specific for the parasites by targeting unique domains of parasite scaffold proteins, LACK (*Leishmania*'s receptor for activated C-kinase) and TRACK (*Trypanosoma* receptor for activated C-kinase).

LACK is a scaffold protein required for the viability of the parasite and for its establishment in the host (Gomez-Arreaza et al., 2011). LACK plays an important role in the early phase of *Leishmania* infection (Mougneau et al., 1995); LACK-deficient parasites are not viable (Kelly et al., 2003), and parasites expressing lower levels of LACK fail to parasitize even immune-compromised mice (Kelly et al., 2003). Similarly, TRACK in *Trypanosoma* is an essential protein and its homologs are found in several trypanosomatids, including *T. cruzi* (Rothberg et al., 2006). Although there is limited information about TRACK's functions in *T. cruzi*, in *Trypanosoma brucei* (*T. brucei*), TRACK is expressed throughout the parasite's life cycle and has a role in the final stages of mitosis (Rothberg et al., 2006). In addition, decreased TRACK expression by RNAi leads to incomplete cytokinesis in pro-cyclic blood stream trypanosomes and accelerates the elimination of parasites from peripheral blood (Rothberg et al., 2006). These data suggest that LACK and TRACK are involved in essential parasite processes and could be important anti-parasitic drug targets.

Previously we developed a rational approach to generate inhibitors of scaffold proteins, which interfere with normal and pathological signaling events in cells, in animal models, and in humans (review (Churchill et al., 2009; Mochly-Rosen and Qvit,

2010; Qvit and Mochly-Rosen, 2010)). Relevant to this study, we have identified peptides that inhibit the function of RACK (receptor for activated C-kinase), the mammalian homolog of LACK and TRACK. RACK is a ubiquitous and highly conserved scaffold protein (McCahill et al., 2002) that binds several signaling enzymes, all of which are critical for cell survival, growth, and differentiation (Schechtman and Moshly-Rosen, 2001). We rationally designed peptides that interfere with RACK function; these peptides were found to be effective and selective in cells, *in vivo* (in a variety of animal models of human diseases (Inagaki et al., 2003; Kim et al., 2008)), and in clinical trials (Bates et al., 2008).

Here we apply the same rational design for development of peptides that target leishmaniasis and Chagas disease. We describe an inexpensive and fast approach that enabled the identification of novel peptides derived from the parasitic scaffold proteins, LACK and TRACK, as anti-parasitic therapeutic leads. These may ultimately provide the basis for a specific, less toxic, and more convenient treatment for people who suffer from these diseases.

2. Materials and methods

2.1. Sequence alignments

Sequences from different species were aligned using the following proteins: human RACK (P63244), *L. donovani* LACK (Q76LS6), *Leishmania braziliensis* LACK (A4HGXX), *Leishmania pan-amensis* LACK (Q9GUB0), *Leishmania major* LACK (Q253306), *Leishmania turanica* LACK (496205235), *Leishmania aethiopica* LACK (496205233), *Leishmania tropica* LACK (404515577), *Leishmania gerbilli* LACK (388850676), *Leishmania infantum* LACK (P62884), *Leishmania chagasi* LACK (P62884), *Leishmania mexicana* LACK (Q7KFG4), *L. amazonensis* LACK (Q95NJ3), *Trypanosoma cruzi* TRACK (Q4DTN2), *Trypanosoma brucei* TRACK (P69103), *Trypanosoma congolense* TRACK (O96653), *Trypanosoma carassii* TRACK (A6ZIC2) and *Trypanosoma vivax* TRACK (O96654). The alignment was done using the FASTA server of the University of Virginia (Pearson and Lipman, 1988), where (:) represents identical amino acids, and (.) represents similar amino acids.

2.2. Peptide synthesis

In brief: Peptides were synthesized on solid support using a fully automated microwave peptide synthesizer (Liberty, CEM Corporation). The peptides were synthesized by SPPS (solid phase peptide synthesis) methodology (Merrifield, 1963) with a fluorenylmethoxycarbonyl (Fmoc)/tert-butyl (tBu) protocol. The lysine side chain was protected with N-methyltrityl (Mtt), a protection group that can be deprotected selectively using acid labile conditions (Aletras et al., 1995). After completion of the synthesis of the linear peptide, an anhydride spacer was coupled to the N-terminal amino group and cyclization was performed using amide bonds between the moiety linker at the backbone N-terminus and an epsilon amino on the side chain of a C-terminal Lys residue (Gilon et al., 1991; Qvit, 2011, 2014). The final cleavage and side chain deprotection was done manually without microwave energy. Peptides were analyzed by analytical reverse-phase high-pressure liquid chromatography (RP-HPLC) (Shimadzu, MD, USA) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) and purified by preparative RP-HPLC (Shimadzu, MD, USA).

Further details: All commercially available solvents and reagents were used without further purification. Dichloromethane (DCM), N-methyl-2-pyrrolidone (NMP), triisopropylsilane (TIS), N,N-diisopropylethylamine (DIEA), O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), 1-

hydroxybenzotriazole (HOBt) and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich (MO, USA); dimethylformamide (DMF) was purchased from Alfa Aesar (MA, USA); piperidine was purchased from Anaspec (CA, USA); rink amide AM resin was purchased from CBL Biopharma LLC (substitution 0.49 mmol/g, CO, USA); Fmoc-protected amino acids were purchased from Advanced ChemTech and GL Biochem (KY, USA and Shanghai, China). Side chains of the amino acids used in the synthesis were protected as follows: Boc (Lys/Trp), But (Ser/Thr/Tyr), OBut (Asp/Glu), Pbf (Arg), Mtt (Lys) and Trt (Asn/Cys/Gln/His).

Peptides were chemically synthesized using Liberty Microwave Peptide Synthesizer (CEM Corporation, Matthews, NC, USA) on solid support with an additional module of Discover (CEM Corporation, Matthews, NC, USA) equipped with fiber-optic temperature probe for controlling the microwave power delivery following the fluorenylmethoxycarbonyl (Fmoc)/tert-butyl (tBu) method in a 30 ml teflon reaction vessel. Each deprotection and coupling reaction was performed with microwave energy and nitrogen bubbling.

Fmoc deprotection was performed in two steps: 30 s and 180 s, both at 45 W, 75 °C using piperidine (20%) in DMF with HOBt (0.1 M) solution. Coupling reactions were performed by repetition of the following cycle conditions: 300 s, 25 W, 75 °C, with HBTU (0.11 M) in DMF, amino acids (0.12 M) in DMF and DIEA (0.25 M) in NMP solution. The coupling and Fmoc deprotection steps were monitored using Kaiser (ninhydrin) Test (Kaiser et al., 1970) and small cleavage. Anhydride coupling was carried out as follows: 300 s, 25 W, 75 °C, using 10:10:1 anhydride/Di-isopropylethylamine (DIEA)/4-dimethylaminopyridine (DMAP) in NMP. Mtt deprotection was carried out using 1:5:94 TFA/TIS/DCM. Cyclization was performed in dibromomethane (DBM) (300 s, 25 W, 75 °C, using 5:10 benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP)/Di-isopropylethylamine (DIEA)). Peptide cleavage from the resin and deprotection of the amino acid side chains were carried out with TFA/TIS/H₂O/phenol solution (90:2.5:2.5:5 v/v/v/w) for 3 h at room temperature without microwave energy. The crude products were precipitated with diethyl ether, collected by centrifugation, dissolved in H₂O/CH₃CN and lyophilized.

Products were analyzed by analytical reverse-phase high-pressure liquid chromatography (RP-HPLC) (Shimadzu LC-20 equipped with: CBM-20A system controller, SPD-20A detector, CTO-20A column oven, 2 × LC-20AD solvent delivery unit, SIL-20AC auto-sampler, DGU-20A5 degasser from Shimadzu, MD, USA) using an ultra 120 5 µm C18Q (4.6 mm ID 150 mm) column (Peeke Scientific, CA, USA) at 1 mL/min. The solvent systems used were A (H₂O with 0.1% TFA) and B (CH₃CN with 0.1% TFA). A linear gradient of 5–50% B in 15 min was applied and the detection was at 215 nm.

The synthesis products were purified by preparative RP-HPLC (Shimadzu LC-20 equipped with: CBM-20A system controller, SPD-20A detector, CTO-20A column oven, 2 × LC-6AD solvent delivery unit and FRC-10A fraction collector from Shimadzu, MD, USA), using an XBridge Prep OBD C18 5 µm (19 mm × 150 mm) column (Waters, MA, USA) at 10 mL/min. The solvent systems used were A (H₂O with 0.1% TFA) and B (CH₃CN with 0.1% TFA). For separation, a linear gradient of 5–50% B in 45 min was applied and the detection was at 215 nm.

Peptides were conjugated to TAT_{47–57} (YGRKKRRQRRR) carrier peptide through an amide bond and with different spacers, as part of the solid phase peptide synthesis. Note that TAT_{47–57}-based delivery of a variety of peptide cargoes into cells has been used now for over 25 years and delivery of the cargo across biological membranes and into subcellular organelles has been confirmed (Begley et al., 2004; Lonn and Dowdy, 2015; Rizzuti et al., 2015). Furthermore, TAT_{47–57} and other cell-penetrating peptides have also successfully been used to deliver cargoes into several

intracellular parasites (Corradin et al., 2002; Dhawan et al., 2003).

2.3. Peptide stability

The stability of the peptides was determined by HPLC using trypsin, which cleaves peptide bonds after lysine and arginine. Peptide (1 mg/mL, 400 µL) was dissolved in NH₄HCO₃ buffer (pH 8.0, 200 mM), and was mixed with a trypsin solution (1 µL, 1 mg/mL in NH₄HCO₃, pH 8.0) (Promega, WI, USA). The peptide was incubated at 37 °C, and samples (100 µL) were taken after 20 min, and every hour. A solution of 2% TFA and 5% ACN in water (100 µL) was added, and the samples were analyzed by HPLC and MS (Pakkala et al., 2007).

2.4. *Leishmania donovani* promastigote viability in culture assay

To evaluate the bioactivity of the peptides, logarithmic phase *L. donovani* promastigote forms ($\Delta aah/\Delta hprt/\Delta xprt$) were seeded in 96-well microtiter plates at 20,000 cells/100 µL in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, NY, USA) media. Promastigotes parasites were incubated with or without peptides (1, 5, 10, 25, 50, 75 and 100 µM) and the culture was incubated for 24 h at 26 °C. The viability of parasites was assessed by adding 20 µL of the vital dye Alamar blue (Fisher Scientific, Ottawa, ON) to each well and cultures were incubated for an additional 24 h at 26 °C; the reduction of Alamar blue was determined by measuring fluorescence at an excitation wavelength of 570 nm and an emission wavelength of 590 nm. All assays were performed in duplicate with the observer blinded to the experimental conditions. Cytotoxicity was expressed as percent survival of control cultures incubated in the absence of peptide. Data are expressed as mean ± S.E. Statistical analysis was assessed by unpaired Student's t-test. A value of $p < 0.05$ was considered significant.

2.5. *Leishmania amazonensis* promastigote viability in culture assay

Cell viability was evaluated *in vitro* by cultivating *L. amazonensis* promastigotes (5×10^6 per well) in M199 medium (Sigma, MO, USA), supplemented with 10% heat-inactivated fetal calf serum (FCS; Invitrogen, CA, USA). Parasites were incubated with or without peptides (1, 5, 10, 25, 50, 75 and 100 µM) at 25 °C for 24 h. Quantification of viable cells was assessed either by cell counting or by measuring the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, MO, USA) as previously described (Zauli-Nascimento et al., 2010). MTT cleavage was assessed in a microplate reader (POLARstar Omega, BMG Labtech, Ortenberg, Germany) with a reference wavelength of 690 nm and a test wavelength of 595 nm. Cytotoxicity was expressed as percent survival of control cultures incubated in the absence of peptide. Data are expressed as mean ± S.E. Statistical analysis was assessed by unpaired Student's t-test. A value of $p < 0.05$ was considered significant.

2.6. *Trypanosoma cruzi* trypomastigote viability in culture assay

To validate the bioactivity of the peptides as anti-parasitic treatment, we used a trypomastigote Y strain (2×10^6) that was obtained from the 5th or 6th day of infection of cultures of the Rhesus Monkey kidney epithelial line (LLC-MK2, ATCC, Boulevard Manassas, VA, USA) (Andrews and Colli, 1982). The assay was carried out in fresh modified Eagle's medium (MEM, Life Technologies, NY, USA) phenol red-free with 2% fetal bovine serum (FBS, Wisent, Montreal, QC) with or without peptides (1, 5, 10, 25, 50, 75 and

100 μ M) for 24 h at 37 °C and 5% CO₂. Parasite viability was measured by a colorimetric assay using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-1) reagent according to manufacturer's instructions (Roche, IN, USA) and measured at 450 nm, and expressed as a percentage of control with no treatment. To corroborate the viability assay, the disruption of the trypomastigotes (parasites) motility was monitored by microscopic examination. Data are expressed as mean \pm S.E. Statistical analysis was assessed by unpaired Student's t-test. A value of $p < 0.05$ was considered significant.

2.7. *Trypanosoma cruzi* epimastigotes viability in culture assay

To validate the potential bioactivity of the peptides on *T. cruzi* epimastigote forms, *T. cruzi* epimastigotes were axenically cultured in liver infusion tryptose (LIT) media, supplemented with 10% fetal bovine serum (FBS, Wisent, Montreal, QC). Parasites were pelleted by centrifugation at $200 \times g$ for 10 min. Epimastigotes were counted using a hemocytometer (Hausser Scientific, Horsham, PA, USA) and seeded at a density of 1×10^7 parasites per well in LIT media in sterile 96-well flat-bottom plates (Becton Dickinson Falcon, Franklin Lakes, NJ, USA). Peptides (1, 5, 10, 25, 50, 75 and 100 μ M) were added to each well. Plates were incubated at 27 °C for 24 h. Live parasites were distinguished and counted using trypan blue (Sigma, MO, USA). Percentage of inhibition is expressed as the number of live parasites/total parasites $\times 100$. Data are expressed as mean \pm S.E. Statistical analysis was assessed by unpaired Student's t-test. A value of $p < 0.05$ was considered significant.

2.8. Mammalian cell toxicity assay

To evaluate the toxicity of the peptides, Rhesus Monkey kidney epithelial cells (LLC-MK2, ATCC, Boulevard Manassas, VA, USA) were seeded in 96-well plates at a density of 8,000 cells/well 24 h prior to treatments. All cell treatments were carried out in fresh modified Eagle's medium (MEM, Life Technologies, NY, USA) medium phenol red-free supplemented with 2% fetal bovine serum (FBS, Wisent, Montreal, QC). The cells received four doses of peptides (200 μ M each) at time-points of 0, 4, 8 and 12 h. After 24 h cell infection was determined by the number of cells and parasites using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-1) reagent according to manufacturer's instructions (Roche, IN, USA) measured at 450 nm, and expressed as a percentage of control with no treatment. Cell morphology was assessed by light microscopy. Data are expressed as mean \pm S.E. Statistical analysis was assessed by unpaired Student's t-test. A value of $p < 0.05$ was considered significant.

2.9. In vivo peptide toxicity assay

BALB/c mice were treated with different peptides (or control, eight mice per group) to assess their toxicity. All treatments were performed between 9:00 a.m. and 4:00 p.m. by an experimenter blinded to the treatment groups. BALB/c female mice (23–26 g, six to eight weeks old), were housed in a temperature- and light-controlled room for at least three days before use. All animals were randomized and assigned to testing groups to generate biological replicates for each group.

Osmotic pumps (#2002, 0.5 μ l/h, Alzet, CA, USA) filled with the p4d (5 mg/kg/day) or vehicle were implanted subcutaneously on the back of the mice following anesthesia using a standard surgical procedure as recommended by the manufacturer.

2.10. *Trypanosoma cruzi* in vivo assays

The study objective tested whether the peptides regulated the level of parasitemia and survival in rodent models. BALB/c mice were infected with *T. cruzi* and treated with different peptides (or controls, five to eight mice per group), to assess their effect on *T. cruzi* infection *in vivo*. The level of parasitemia was measured every third day and survival was recorded.

Based on our experience, a minimum of five rodents per group is required to obtain statistically meaningful data. An experimental group size of five or more animals is necessary to achieve at least a 20% minimal difference in parasitemia and survival for a power of 95% with $\alpha < 0.05$ and $\beta < 20\%$. All treatments were performed between 9:00 a.m. and 4:00 p.m. by an experimenter blinded to the treatment groups. BALB/c female mice (23–26 g, six to eight weeks old), were housed in a temperature- and light-controlled room for at least three days before use. All animals were randomized and assigned to testing groups to generate biological replicates for each group.

In the first study, BALB/c mice (five mice per group) were treated with peptides by intraperitoneal (*ip*) injection for 7 consecutive days and after two days (day 10) given an additional injection for a total of 8 injections of p4d (1.5 mg/kg/day) or vehicle. One day before peptide treatment started, mice were infected with 500 bloodstream trypomastigotes (Strain H1 of *T. cruzi*) *via ip* injection.

In the second study osmotic pumps (#2002, 0.5 μ l/h, Alzet, CA, USA) filled with p4d (1.5 mg/kg/day) or vehicle were implanted subcutaneously on the backs of the mice under anesthesia using a standard surgical procedure as recommended by the manufacturer. One day following pump implantation, mice were infected with 500 bloodstream trypomastigotes (Strain H1 of *T. cruzi*) *via intraperitoneal* injection. One group of control mice received Benznidazole (Bz) (Sigma, MO, USA, 100 mg/kg/day) administered orally for 20 days starting on day 1 post-infection.

2.11. Parasitemia level and mortality

Parasitemia in the blood was measured every third day in infected mice by microscopic counting using a Neubauer chamber, and survival was recorded daily for up to 24 days (first study) or 50 days (second study) post-infection (Limon-Flores et al., 2010) with the observer blinded to the experimental conditions.

2.12. *T. cruzi* parasite burden post mortem

Parasite burden was determined by quantitative real-time PCR (qPCR) amplification of *T. cruzi* DNA from cardiac biopsies (Quijano-Hernandez et al., 2013). Briefly, DNA was purified from cardiac biopsies with QIAamp DNA Mini Kit (Qiagen, Limburg, Netherland) following the manufacturer's instructions. A standard curve was generated using mouse DNA spiked with known serial dilutions of *T. cruzi* DNA. PCR reactions contained 50 ng of mouse DNA, 0.5 μ M primers TCZ-F 5'-GCTCTTGCCACAMGGGTGC-3' and TCZ-R 5'-CCAAGCAGCGGATAGTTCAGG-3', and 10 μ L of EXPRESS SYBR GreenER™ qPCR Supermix (Invitrogen, CA, USA) in a final volume of 20 μ L. Reactions were run in triplicate on an Eco™ Real-Time PCR System (Illumina, CA, USA) as follows: 50 °C for 2 min and 40 cycles of 95 °C for 10 s, 55 °C for 15 s and 72 °C for 5 s. High-resolution melting curves were determined at the end of the amplification. The standard curve had a slope of -3.127 , $R^2 = 0.9932$ and an efficiency of 109%.

2.13. Statistical methods

Data are expressed as mean \pm S.E. Statistical analysis was

assessed using the two tailed unpaired Student t-test, one-way or two-way measures analysis of variance (ANOVA) with post-hoc testing by Tukey when appropriate. A value of $p < 0.05$ was considered significant. At least three independent experiments were performed for each data set. Sample sizes for in culture experiments were estimated based on previous experience of similar assays and the effect size observed in preliminary experiments. No samples were excluded from analysis. All samples were identical prior to allocation of treatments and the observer was blinded to the experimental conditions.

2.14. Animal care

Animal care and husbandry procedures were in accordance with established institutional and National Institutes of Health guidelines. The animal protocols were approved by the Ethical Committee of the Universidad Autónoma de Yucatán, Mexico, and by the Stanford University Institutional Animal Care and Use Committee.

2.15. Secondary structure studies, Raman experiments

In the Raman spectra, the shape of amide I and amide III bands were analyzed to determine the secondary structure of the biomolecules. The shape of amide III band was evaluated qualitatively, while the deconvolution of the amide I was performed as follows (Maiti et al., 2004): 5 Lorentzian peaks for α -helix (1651 cm^{-1}), β -sheet (1668 cm^{-1}), random coil (1683 cm^{-1}), tyrosine peak at 1620 cm^{-1} and exponential baseline (represented with a broad Lorentzian peak centered at 1500 cm^{-1}). Raman experiments were performed with NTEGRA Spectra Raman spectrophotometer instrument, equipped with a peltier plate-cooled CCD camera in backscattering geometry. The illumination source was a 473 nm Cobolt Blues™ continuous wave diode-pumped solid state laser brought to the sample by a Mitutoyo long working distance objective (100 \times , 0.7 NA). Laser power at sample was 2 mW, as measured by Coherent LaserCheck™. Samples were measured in dry state. Spectra from dry samples were acquired in 1 min increments for 10 min total. The absence of sample degradation was confirmed by absence of any difference between the 1-min spectra, besides minimal changes in fluorescence baseline signal.

3. Results

3.1. Rational design of peptides with anti-parasitic activity derived from LACK and TRACK

We hypothesized that short sequences in the scaffold proteins LACK and TRACK that are conserved among parasite strains and species, but are not conserved in the mammalian RACK, may mediate interactions important to the parasites but not to the mammalian host. We focused on LACK in *Leishmania* and compared it to RACK, the ortholog in mammalian hosts (Fig. 1). Although LACK is similar (>75% similar and 48% identical) to RACK, at least eight distinct regions in LACK differ substantially ($\leq 57\%$ similarity and $\leq 30\%$ identity to RACK; Fig. 1A; these regions are designated as L1 to L8). Importantly, these eight regions in LACK are conserved between different species of *Leishmania* (Supplementary Figure 1), suggesting that they may be functionally important for the parasite. Most of the non-conserved regions between RACK and LACK are also less conserved between RACK and TRACK (Supplementary Figure 2) and are conserved between different *Trypanosoma* species (Supplementary Figure 3). We hypothesized that peptides derived from these parasite-unique regions may have anti-parasitic activity without affecting mammalian host signaling. We

synthesized peptides (p1–p8) derived from regions L1–L8 in LACK (Supplementary Figure 4–5 and Table 1) and tested them for their bioactivity towards parasites.

We also covalently linked the peptides to a cell-penetrating peptide (CPP), which allows the peptides to cross cell membranes (Lonn and Dowdy, 2015). Peptides conjugated to CPP have previously been delivered intracellularly to the parasites *Leishmania* and *Plasmodium* (the parasite causing malaria), where they inhibit infections (Corradin et al., 2002; Dhawan et al., 2003). Specifically, we used TAT_{47–57} (YGRKKRRQRRR, Supplementary Figure 4), which has been shown to deliver macromolecules into cells in cell culture and in animal disease models *in vivo*, and which has been used in over 25 clinical trials (Lonn and Dowdy, 2015).

3.2. LACK-derived peptides show anti-parasitic activity

Treatment of *L. donovani* promastigotes with peptide p4a (see Supplementary Table 1 for sequence information) reduced the parasite viability in culture by 60% at 48 h (Fig. 2A, left panel; IC₅₀ ~40 μM , Fig. 2B, dashed lines), and p3 treatment resulted in ~50% reduction of parasite viability (Fig. 2A). None of the other targeted peptides had any effect on *L. donovani* promastigote viability. In addition, treatment with p4a reduced the viability of *L. amazonensis* promastigotes in culture by ~100% after 24 h (IC₅₀ ~23 μM , Fig. 2B, solid lines). TAT peptide alone and p4, which lacks TAT (Supplementary Table 1), showed no anti-parasitic activity (Fig. 2A–B), demonstrating that TAT is not cytotoxic and that intracellular delivery by TAT is required for the anti-parasitic effect of the cargo.

The L4 region in LACK has 88% similarity to TRACK (Fig. 2C). Treatment of *T. cruzi* trypomastigotes in culture with the LACK-derived peptide, p4a, reduced the viability of the parasites by ~75% compared with control (Fig. 2A, right panel; IC₅₀ ~20 μM , Fig. 2D). Again, the TAT or p4 alone showed no anti-parasitic activity (Fig. 2A, D).

3.3. Structure–activity relationship studies of p4a

We tested the effect of the relative positions of the cargo (p4) and the carrier (TAT), and the effect of different linkers between the carrier and the cargo on the leishmanicidal (*L. donovani*) and trypanocidal (*T. cruzi* epimastigotes) activity (Fig. 3B and Supplementary Figure 5 show peptide structures). When the cargo was linked at the C-terminus of TAT by a flexible GG linker (p4b, Supplementary Table 1 and Fig. 3B), the anti-parasitic effect was completely lost relative to a peptide with the same linker and the cargo at the N-terminus (p4a vs. p4b; Fig. 3A). In contrast, when the cargo was linked at the TAT C-terminus using a constrained gamma amino butyric acid (GABA; p4c) linker, the anti-parasitic activity was comparable to that observed in the reverse order using the flexible GG linker (p4a; Fig. 3A).

Conformational constraints such as cyclization usually decrease degradation and increase bioactivity of peptides, as compared with their linear counterparts (Qvit et al., 2008; Qvit and Crapster, 2014; Qvit and Kornfeld, 2016). Therefore, we developed two cyclic peptides and tested their activity in the same assay. One of the two cyclic peptides, p4d, was more active than the linear peptide, p4a, reducing parasite viability to only ~5% as compared with control (Fig. 3A). The IC₅₀ of p4d was 4–5 fold lower than the IC₅₀ of p4a (IC₅₀ ~6 μM and IC₅₀ ~3 μM for *L. donovani* and *T. cruzi* epimastigotes, respectively, Fig. 3C; vs. IC₅₀ of ~20 μM for both by p4a, Fig. 2B, D). Importantly, the peptides derived from the L4 region did not affect the viability of naive LLC-MK2, Rhesus monkey kidney cells, confirming that the peptides are not toxic to the mammalian host cells (Fig. 3D).

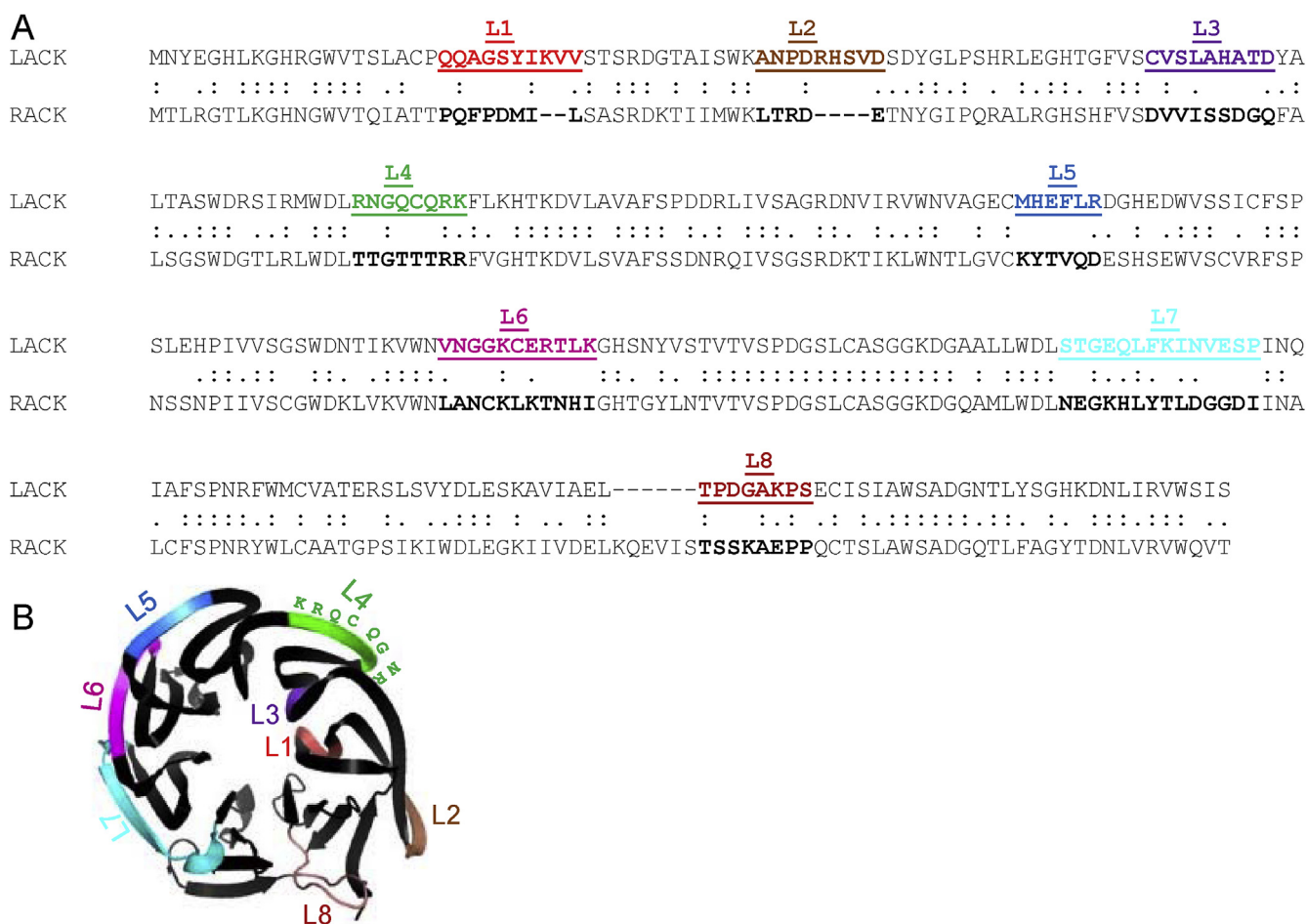


Fig. 1. Rational design of peptides with anti-parasitic activity. Sequence alignment between human RACK (P63244) and *L. donovani* LACK (Q76LS6). (A) The alignment identified eight short regions labeled L1–L8 of low similarity between LACK and RACK. The alignment between the proteins was done using the FASTA server, where: (:) represents identical amino acids, and (.) represents similar amino acids. (B) The position of identified L regions in A are highlighted in the structure of the *Trypanosoma* LACK homolog, TRACK (PDB: 4V8M) and colored as in A. We used the TRACK structure because the structure of LACK is not available and there is high similarity between LACK and TRACK (over 75% similarity). The sequence of the L4 region in LACK is also provided on the structure.

We tested the stability of the peptides to determine if the increased activity of the cyclic peptide is due to its improved resistance to degradation. The linear peptide p4a was less stable (>55% degradation by trypsin occurred in <20 min, Fig. 4A–B) than the cyclic peptide p4d (<20% trypsin cleavage in 6 h, Fig. 4C–D). The increased stability may account for the superior anti-parasitic activity of the cyclic peptide compared to the linear peptide. Importantly, despite the increased constraint in p4d relative to p4a, analysis of the secondary structure of these peptides using Raman spectroscopy showed that their structures were very similar (Supplementary Figure 6). Amide III regions did not exhibit a pronounced peak, suggesting that the majority of the structure was disordered for all three molecules. The amide I peak showed a mixture of β -sheet and unordered secondary structures. The p4a and p4d molecules showed a higher percentage of ordered structure, mostly due to an increase in α -helix content. In the figure, the height of the tyrosine peak is compared to the height of the amide I band (pairs of horizontal dashed lines). It can also be seen that the ratio of the intensities of Tyr and amide band slightly increases as follows: TAT < p4a < p4d, which is due to an increase in the number of amide bonds per molecule. This proves that the measured conformations come mostly from the cargo amino acids and not solely the carrier.

Structure–activity relationship (SAR) studies on the cargo of the

cyclic peptide helped determine which amino acids in the peptide are required for bioactivity. Alanine scanning of the cyclic peptide p4d demonstrated that substitution of any of the amino acids from the N-terminal half (R, N, G, or Q) with alanine (A) only slightly reduced the anti-parasitic activity of the peptide. However, substitution of any one of the amino acids in the C-terminal half (C, Q, R or K) caused a reduction or a complete loss of anti-parasitic activity, indicating that the C-terminal half of the peptide is required for this activity (Fig. 5A). Interestingly, the same C-terminal half is the most highly conserved in the L4 region between LACK and TRACK (Fig. 5B), which may explain why the L4 region-derived peptide (from LACK) had both leishmanicidal and trypanocidal activity.

3.4. p4d decreases parasite burden in mice infected with *T. cruzi*

We performed two preliminary studies to evaluate the effect of the peptides in mice infected with *T. cruzi*. The first study was designed as a fast screen (24 days). BALB/c mice (five females per group) were infected with trypomastigotes (*T. cruzi*) via intraperitoneal (*ip*) injection, and on the following day we began the peptide treatment. Mice were treated with 8 *ip* injections of p4d (1.5 mg/kg/day) or vehicle. Treatment with p4d reduced the parasitemia by 85% (day 7) and 80% of the mice survive compared to none of the vehicle treated mice (day 24) (Supplementary Figure 7). Next we

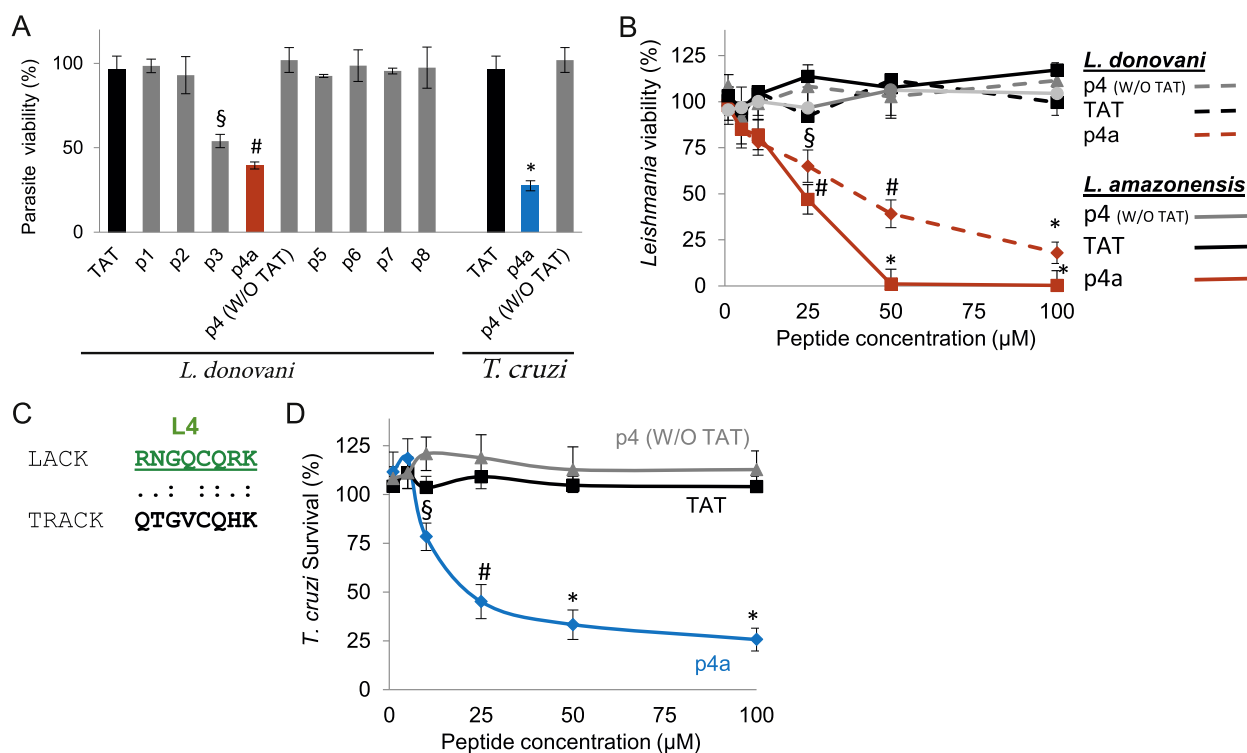


Fig. 2. Leishmanicidal and trypanocidal activity of peptides in culture. Peptides were added to *L. donovani* promastigotes as a single addition in DMEM media, or to trypomastigote forms of *T. cruzi* cultured in LLC-MK2 Rhesus monkey kidney epithelial cells, and their leishmanicidal and trypanocidal activity was evaluated after 48 or 24 h, respectively. See Materials & Methods sections 2.5 and 2.7 for more experimental details. (A) Anti-parasitic activity was assessed with various peptides derived from various domains in LACK, p1–p8, and conjugated to TAT carrier (TAT_{47–57} (Rizzuti et al., 2015)) (given once, at 100 μM, each, red - leishmanicidal activity and blue - trypanocidal activity). Only p3 and p4a had leishmanicidal activity (Left panel). p4a also had high trypanocidal activity (Right panel). (B) Dose-response curve of p4a against *L. donovani* promastigotes (48 h) and *L. amazonensis* promastigotes (24 h). p4a had leishmanicidal activity [IC₅₀ ~40 μM (dash lines) and IC₅₀ ~23 μM (solid lines) against *L. donovani* promastigotes and *L. amazonensis* promastigotes, respectively]. (C) Sequence alignment of L4 region between *L. donovani* LACK (Q76LS6) and *T. cruzi* TRACK (Q4DTN2). L4 region is 50% identical and 88% similar between LACK and TRACK, where: (:) represents identical amino acids, and (.) represents similar amino acids. (D) Dose-response curve of p4a, p5 and TAT activity against *T. cruzi*. p4a had trypanocidal activity (IC₅₀ ~20 μM against *T. cruzi*, 24 h). Data are from three experiments expressed as mean ± SEM, and the differences in susceptibility were statistically significant ([§]p < 0.01, [#]p < 0.05 and ^{*}p < 0.001 vs. TAT), as assessed by Student's t test. The observer was blinded to the experimental conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

performed a second study, using eight females per group, and followed the mice for 50 days. Mice were infected with trypomastigotes (*T. cruzi*) via *ip* injection and were treated with p4d or vehicle for the first 14 days after infection at 1.5 mg/kg/day, using osmotic pumps, which provide slow and sustained delivery of the peptide. In this study, p4d decreased parasitemia by more than 75% (days 39–42) (Fig. 6A). The treatment also increased host survival by over three-fold (Fig. 6B), and reduced parasite burden in cardiac tissue by three-fold (Fig. 6C).

We performed an additional animal study to evaluate the toxicity of p4d. Osmotic pumps were used to deliver p4d for 28 days at 5 mg/kg/day, a 3.3 fold higher dose than in the anti-parasitic study, above. The effect of the prolonged treatment on mice was compared to that of vehicle-treated mice (eight BALB/c females per group). The peptide did not show any toxic effects (there was no weight difference, induction of seizures, strange behavioral or metabolic disorders in the p4d relative to vehicle-treated mice) when the naive uninfected mice were treated with >3× the dose for twice the duration as the infected mice.

In conclusion, in both animal parasite models, p4d reduced the level of parasitemia by >65%, increased the survival by at least three-fold, and decreased the parasite burden on the peak days. In the second study (50 days) one control group was treated with Benznidazole (Bz) (Fig. 6A) for a longer duration than the peptide (21 days vs. 14 days, respectively) and with much higher dose (100 mg/kg/day vs. 1.5 mg/kg/day, respectively; moreover since the

molecular weight of Benznidazole is less than 10% of p4d (216.25 g/mol vs. 2854.32 g/mol, the mole ratio between Benznidazole and p4d is 920:1 (0.46 mmol vs. 0.0005 mmol)). In this group parasitemia was undetectable and all the mice survived the study. These combined data confirm the cell culture data and suggest that further optimization of peptide sequence, formulation, dose, and route of administration should achieve higher efficacy *in vivo*.

4. Discussion

Neglected tropical diseases, endemic in developing regions of the world, are responsible for significant morbidity among low-income populations (Hotez et al., 2007). Most current treatments for these diseases are toxic and are limited by drug resistance. Toxicity occurs because most drugs are relatively non-selective toxins with more generalized mechanisms of action affecting both parasite and host. Many drugs are directed to the catalytic site of enzymes such as proteases, kinases, and metabolic enzymes ((Olin-Sandoval et al., 2010); for review (Qvrit and Crapster, 2014; Renslo and McKerrow, 2006)). Although there has been some success using this approach for the treatment of Chagas' disease, such as K777 (an α-ketone irreversible cruzipain inhibitor) (Barr et al., 2005), these catalytic sites are usually highly conserved between the parasites and mammalian hosts, and therefore it is challenging to specifically target the parasite (Seebeck et al., 2011). The other limitation of current treatments relates to drug

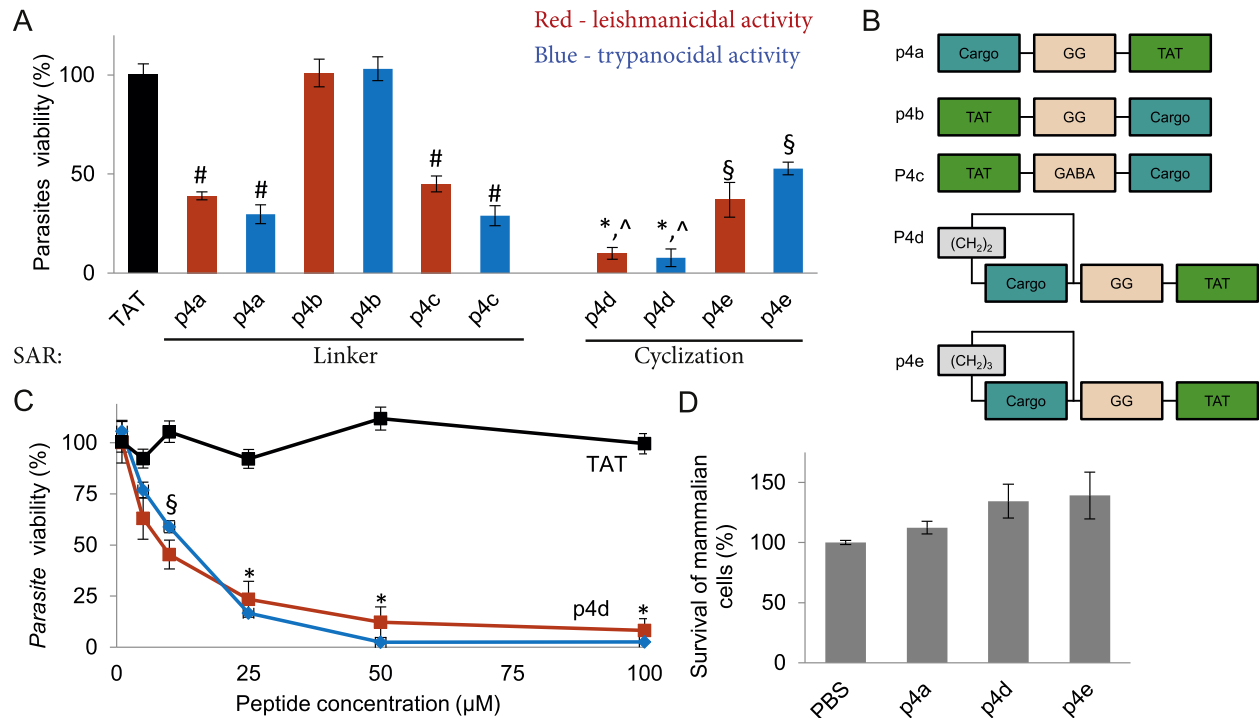


Fig. 3. Structure activity relationship (SAR): Studies of the linker and cyclization of the peptides in culture. Peptides were added once to *L. donovani* promastigotes or to epimastigote forms of *T. cruzi*, and their leishmanicidal and trypanocidal activity was evaluated after 48 or 24 h, respectively. See Materials & Methods Sections 2.5 and 2.7 for more experimental details. (A) Anti-parasitic activity was assessed with various peptide analogs having modified linkers between the cargo and the TAT carrier (TAT₄₇₋₅₇ (Rizzuti et al., 2015)) and changing the position of the TAT carrier relative to the cargo, as well as p4a analogs having conformational constraints (given once, at 100 μM, each, red - leishmanicidal activity and blue - trypanocidal activity). (B) Peptides schematic structure. The peptides are comprised of TAT₄₇₋₅₇ (Rizzuti et al., 2015) (green); linker (light brown), one or two amino acids used as linker between TAT and the cargo; and p4, the cargo (blue). Cyclic peptide analogs with conformational constraints of p4 were prepared; these analogs have the same amino acids as the linear analog and an extra alkyl chain (grey). (C) Dose-response curve of p4d against *Leishmania* (red) and *T. cruzi* (blue) parasites. p4d has leishmanicidal (*Leishmania donovani*) and trypanocidal (*T. cruzi* epimastigotes) activity was evaluated after 48 or 24 h, respectively. (IC₅₀ ~6 μM and IC₅₀ ~3 μM, respectively). (D) Incubation with the p4 analogous peptides did not affect the viability of naïve (non-infected) LLC-MK2 cells, indicating that the peptides are not toxic to the mammalian host (100 μM, delivered 4 times over 24 h). The observer was blinded to the experimental conditions. Data are representative of three independent experiments and presented as mean ± SEM. Statistical analysis was performed using two-tailed unpaired Student's t-test (*p < 0.01, #p < 0.05, ^p < 0.001 vs. TAT; §p < 0.05 vs. the linear peptide, p4a). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

resistance, which emerges in part because of lack of compliance (i.e., early or inconsistent treatment) may enable development of mutations in the drug target. We have described here a simple and fast approach to identify an anti-parasitic peptide that may overcome the limitations of current treatments in the following ways: (1) Our candidate should have less or no toxicity, because it specifically targets protein domains that are unique to the parasite, not the host; (2) This approach reduces the probability that resistance will arise because substitution mutations in the parasites are much less likely to occur at evolutionarily conserved sites in proteins (Thomas et al., 2003).

We focused our drug discovery efforts on the parasite-specific scaffold proteins, LACK and TRACK, which are essential for the survival and infectivity of both *Leishmania* sp. and *T. cruzi*, respectively (Mouneau et al., 1995; Rothberg et al., 2006; Choudhury et al., 2011). Our rational approach identified an inhibitor which inhibited parasites' growth in culture and *in vivo* without exerting toxic effects. We show that peptides corresponding to the L4 region from LACK, which is only ~20% identical to mammalian RACK (Fig. 1), had anti-parasitic activity against both *Leishmania* sp. and *T. cruzi* in culture and showed anti-trypanosomal activity *in vivo*. Two different animal models were used in these latter studies: a short study (24 days) in which the peptide was delivered by *ip* injection and a longer study (50 days), in which the peptide was delivered in a sustained fashion, using subcutaneous osmotic pumps. Osmotic pumps permit continuous administration of short

half-life molecules and are gaining widespread use for targeting parasites (Shibata et al., 2011). In both studies, the peptide treatment was for a short time (only one-third of the study) and in a low dose (1.5 mg/kg/day). Nevertheless, in these two studies p4d demonstrated promising anti-parasitic activity. The peptide was conjugated to TAT, which is used to deliver large macromolecules and peptides into cells. Furthermore, it was recently shown that when miltefosine, an oral anti-parasitic drug, was linked to TAT, the resulting compound was efficiently internalized into a miltefosine-inulnerable *Leishmania* strain, resulting in fast parasite killing, and successful avoidance of resistance (de la Torre et al., 2014). Therefore, TAT is a proven tool to improve drug intake and treatment efficacy in the parasite. Optimized peptoid delivery using a subcutaneous deposit (single injection) or a sustained delivery employing a subcutaneous osmotic pump (similar to that used for yearly contraceptive delivery in some developing countries) could ensure effective delivery and compliance to drug treatment in humans, especially in rural areas, and therefore decrease the chance of resistance emerging.

Although the biology of *T. cruzi* and *Leishmania* is significantly different, it has already been shown that targeting either unique parasite proteins or exclusive domains on scaffold proteins can be a successful approach for the development of anti-parasite agents; inhibitors targeting several key proteins, such as polyamine biosynthetic enzymes (Heby et al., 2007), trypanothione reductase (Parveen et al., 2005), sterol 24-methyltransferase (Magaraci et al.,

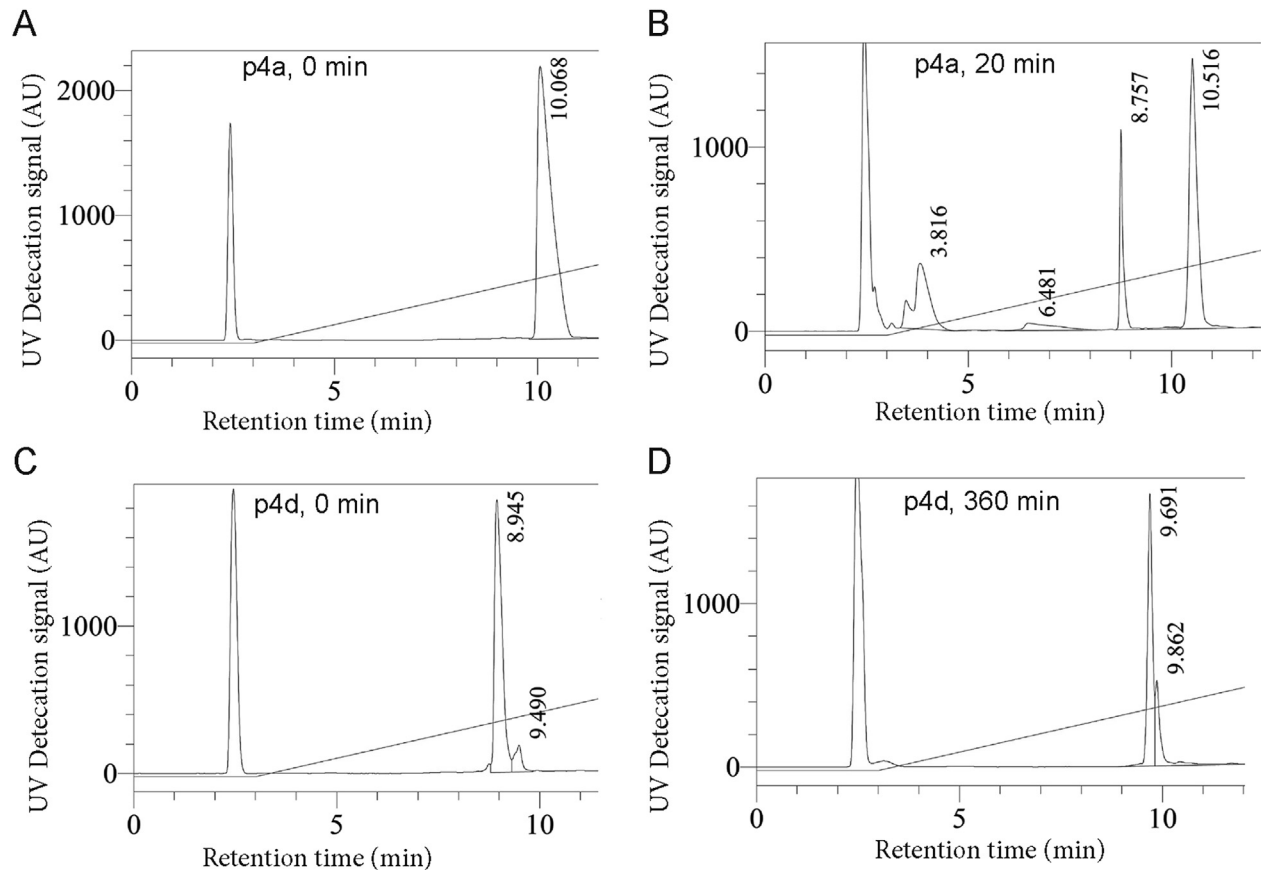


Fig. 4. Stability studies of the native p4a peptide and the cyclic peptide, p4d. Stability of peptides against proteolytic digestions as determined by HPLC analysis using trypsin (for more experimental details, see Section 2.3 Peptide stability). (A–B) Over 55% degradation of the linear peptide, p4a, occurred in less than 20 min (C–D) Proteolytic cleavage of the cyclic peptide (p4d) was much slower, with <20% of the cyclic peptide cleaved in 6 h. The cyclic peptide, p4d, was found to be significantly more stable than the linear peptide, p4a.

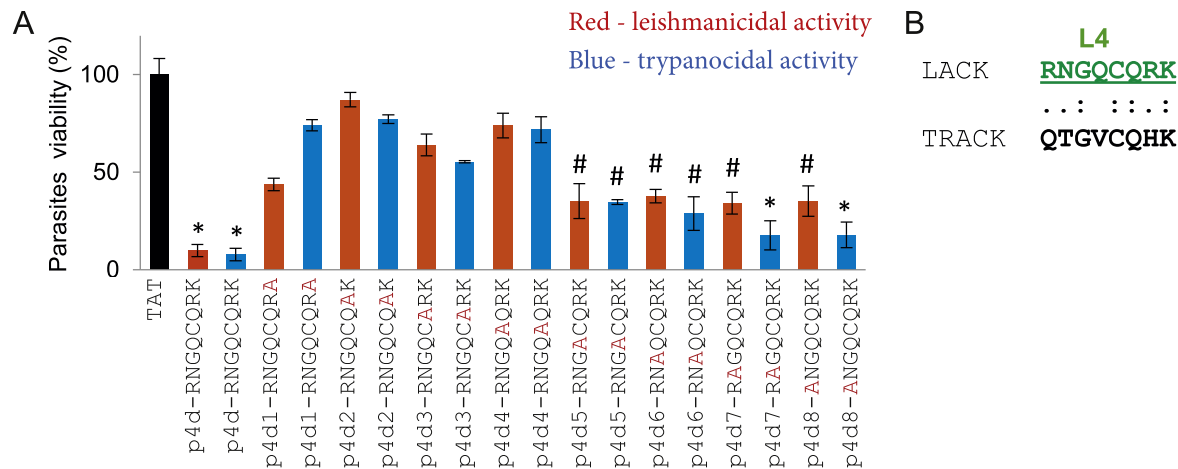


Fig. 5. Structure activity relationship (SAR) studies: Alanine scan of the cyclic peptide in culture. Peptides were added once to *L. donovani* promastigotes or to epimastigote forms of *T. cruzi* and their leishmanicidal and trypanocidal activity was evaluated after 48 or 24 h, respectively. (A) Alanine scan of p4d. Anti-parasitic activity was assessed after treatment with p4d and alanine-scan analogs (given once, at 100 μ M). Substitution of the amino acids from the N-terminal (R, N, G, or Q) only slightly reduced the anti-parasitic activity of the peptide. Though, substitution of any one of the amino acids in the C-terminal half (C, Q, R or K) caused a reduction or a complete loss of anti-parasitic activity. (B) Sequence alignment of L04 region between *L. donovani* LACK (Q76LS6) and *T. cruzi* TRACK (Q4DTN2). L4 region is 50% identical and 88% similar between LACK and TRACK, where: (:) represents identical amino acids, and (.) represents similar amino acids. Data are representative of three independent experiments and presented as mean \pm SEM. Statistical analysis was performed using two-tailed unpaired Student's t-test (* p < 0.05, ** p < 0.001 vs. TAT). The observer was blinded to the experimental conditions.

2003) and others, affect both parasites. In support of our finding that peptides derived from the L4 region of RACK exhibit both anti-leishmanial and anti-trypanosomal effects, SAR studies

demonstrated that amino acids found to be critical for the bioactivity were those conserved between LACK and TRACK. Further studies exploring the bioactivity of L4 region-derived peptoids

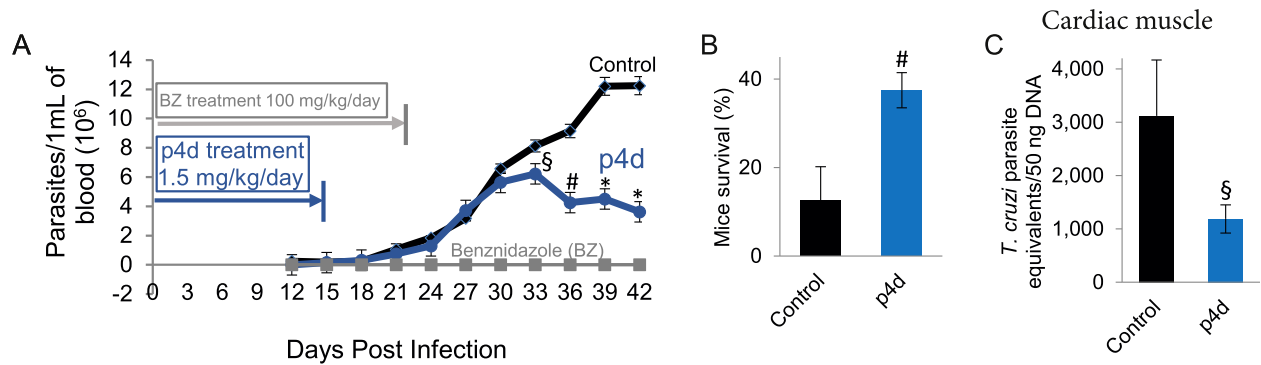


Fig. 6. The effect of p4d on *T. cruzi* infections in vivo. *T. cruzi* parasitemia level, mortality, and parasite burden in infected mice (see Materials and Methods for more experimental details). Mice treated with the cyclic peptide, p4d, showed a 65% reduction in parasitemia (A), increase in survival by three-fold (B) and significantly less parasite burden of amastigotes in the cardiac muscle at the peak days (days 39–42) (C). The observer was blinded to the experimental conditions. Data presented as mean \pm SEM. Statistical analysis was performed using two-tailed unpaired Student's t-test (§ p < 0.01, # p < 0.05, * p < 0.001 vs. control).

against *Leishmania* amastigotes, as well as targeting *T. cruzi* with peptides derived from TRACK, are now under evaluation. In addition, the cysteine from the L4 region of LACK is conserved in most *Leishmania* species as well as between LACK and TRACK. However, because it can be susceptible to rapid oxidation, the anti-parasitic activity of substituting the cysteine with serine, methionine, etc, will be valuable. Moreover, additional considerations should be given to the difference in the biology of each parasite in future optimization studies of this lead peptide, p4d. While *T. cruzi* divides within the cytoplasm, *Leishmania* divides within the phagolysosome, and therefore peptides targeting *Leishmania* need to cross more membranes and act within the acidic environment of the phagolysosome.

Importantly, although peptides that work intracellularly are not traditionally considered to be suitable drugs, this notion is now being challenged in general (Fosgerau and Hoffmann, 2015) and also for anti-parasite agents (Qvit and Crapster, 2014). Previously, we have shown that peptide inhibitors can be useful pharmacological agents. For example, we have used peptide inhibitors of RACK in animal models of heart failure (Mochly-Rosen et al., 2012) and myocardial infarction (Inagaki et al., 2003) as well as in patients (Bates et al., 2008). Although the optimal treatment regimen and dose have not yet been determined, our data suggest that peptoids (modified peptides) derived from the L4 region of LACK, which will be highly specific and effective, will fulfill the target product profile published by DNDi (<http://www.dndi.org/diseases-projects/diseases/chagas/target-product-profile.html> and <http://www.dndi.org/diseases-projects/diseases/vl/tpp/tpp-vl.html>), and therefore will be promising candidates as anti-parasitic lead compounds. In addition, the possibility to combine the peptide lead compound that we identified with other chemotherapy is a valuable option. It may be most effective to try combination with current drugs that have different mechanisms of action, for example benznidazole; such a combination may reduce the potential for resistance as well as decrease the required quantity of benznidazole, thereby reducing toxicity.

Moreover, topical formulations offer several advantages over systemic delivery, including easier administration, minimizing adverse effects and reducing cost (Garnier and Croft, 2002; Moreno et al., 2014). One approach may be to use the peptides as topical treatment for localized CL.

The rational method that we used here to identify an effective drug lead is rapid, does not require high throughput screens or extensive medicinal chemistry efforts, and uses very limited capital. We suggest that this approach to generate inhibitors is likely

generalizable and can be adapted for the design of other novel drugs.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ijpddr.2016.02.003>.

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